HRAD1 and *MRAD1* encode mammalian homologues of the fission yeast *rad1*⁺ cell cycle checkpoint control gene

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ABSTRACT

Eukaryotic cells arrest at the G₂ checkpoint in the presence of DNA damage or incompletely replicated DNA. This cell cycle checkpoint prevents the development and propagation of genomic instability. In the fission yeast, this process requires the action of a number of genes, including rad1+. We report here the identification of human and mouse cDNAs that exhibit extensive sequence homology to rad1+. The human gene, called HRAD1, encodes a 282 amino acid protein that is 27% identical and 53% similar to yeast Rad1p. The human homologue maintains its sequence similarity over the full length of the protein, including the three proposed $3' \rightarrow 5'$ exonuclease domains, and the leucine rich repeat region. The mouse gene, called MRAD1, encodes a 280 amino acid protein that is 90% identical and 96% similar to HRAD1 at the amino acid level. Expression of HRAD1 in yeast rad1 mutants partially restores radiation resistance and G₂ checkpoint proficiency to these mutants. Evolutionary conservation of structure between HRAD1, MRAD1, rad1+, Saccharomyces cerevisiae RAD17 and the Ustilago maydis REC1 checkpoint genes suggests that the function of the encoded proteins is conserved as well. The ability of HRAD1 to partially complement yeast rad1 mutants suggests that this gene is required for G₂ checkpoint control in human cells.

INTRODUCTION

Cell cycle checkpoints are regulatory mechanisms that ensure prerequisite events are completed before subsequent cell cycle transitions occur. For example, mitotic entry is dependent on the prior completion of DNA replication. Checkpoints also exist to prevent the propagation of damaged chromosomes that can result from radiation or radiomimetic drugs. These DNA damage checkpoints operate predominantly at the G₁/S and G₂/M transition points (1).

Even in the absence of exogenous DNA damage or blocked DNA replication, checkpoint mutants are known to exhibit

genomic instability, as seen in RAD9 mutants of Saccharomyces cerevisiae at the G_2/M checkpoint (2), and in $p53^{-/-}$ mammalian cell lines at the G1/S checkpoint (3,4). The accumulation of mutations in cells exhibiting genomic instability has been suggested to be the driving force behind tumour formation and metastasis (5,6). This is supported by studies on individuals with inherited chromosome instability diseases which include ataxia telangiectasia, Li-Fraumeni syndrome and Bloom's syndrome (7-10). In all three cases, genomic instability and cancer predisposition are seen, with the former operating at the cellular level and the latter at the level of the individual. The genes mutated in these diseases are ATM, p53 and BLM, respectively. The ATM protein is a member of the PI-3 kinase family (11,12) and p53 is a transcription factor (13–15), and both are known to have checkpoint functions (16-23). The BLM protein has structural homology with known helicases and is also thought to function in checkpoint control (24,25).

A recent report has shown a strong correlation between loss of the G₂ checkpoint and the appearance of chromosomal abnormalities (26), suggesting that the G_2 checkpoint is a major protective factor against the development of genomic instability and cancer. Despite its apparent importance, only two presumptive components of the mammalian G₂ checkpoint have been identified to date (27-29). By contrast, the G₂ checkpoint has been well characterised in the fission yeast Schizosaccharomyces pombe. Fission yeast undergo a dose dependent G2 delay following exposure to radiation and the resultant DNA damage that occurs (30,31). The yeast remain arrested at G₂ while the damage is repaired, then enter mitosis and resume progression through the cell cycle. This dose dependent response to radiation is absent from mutants of any one of the six checkpoint rad genes rad1⁺, rad3⁺, rad9⁺, $rad17^+$, $rad26^+$ and $hus1^+$ (30–33). Mutants of any one of these genes have similar phenotypes; they are hypersensitive to radiation and to transiently inhibited DNA replication, such as occurs in the presence of hydroxyurea (HU). The sensitivity of these mutants to radiation and HU results from loss of the G2 DNA damage checkpoint and the S phase checkpoint monitoring completion of DNA synthesis, respectively (30-33).

The fission yeast $rad1^+$ gene has previously been shown to be conserved among lower eukaryotes. *Saccharomyces cerevisiae*

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RAD17 (34) and *Ustilago maydis REC1* (35,36) are functional homologues of $rad1^+$. *RAD17* and *REC1* were shown independently to be required for checkpoint function, and both exhibit moderate sequence conservation with $rad1^+$ (25–30% at the amino acid level). We report here the cloning of human and mouse homologues of the *S.pombe rad1*⁺ gene, called *HRAD1* and *MRAD1*, respectively. Expression of *HRAD1* in yeast *rad1* mutants results in partial restoration of the G₂ checkpoint response to radiation. Expression of *HRAD1* in these yeast does not restore resistance to HU. We propose that *HRAD1* and *MRAD1* are components of the G₂ checkpoint mechanism in humans and mice, respectively.

MATERIALS AND METHODS

DDBJ/EMBL/GenBank accession numbers

The accession numbers for the *HRAD1* and *MRAD1* cDNA sequences are AF011905 and AF038841, respectively.

cDNA libraries, screening and sequencing

The HaCaT cDNA library in λ ZAP II was a gift of D.Beach, and the CB7 mouse erythroleukemia cDNA library was a gift from P.A.Greer. A probe for screening the HaCaT cDNA library was generated by amplification of a 399 bp portion of the EST sequence (DDBJ/EMBL/GenBank accession no. AA029300) using primers A (GGTACATGACCTTGCTCCTAT) and B (AGTTCCCACCTTGACTATCC), and HaCaT cDNA as template. The full-length HRAD1 cDNA was used as a probe to screen the mouse cDNA library. Library screens were performed using standard techniques (37). Sequencing of both strands of the HRAD1 and MRAD1 cDNAs was performed on an ABI 377 automated sequencer after subcloning into pBluescript KS-. Amino acid sequence alignments were generated using the CLUSTAL W program (38). In the amino acid alignment, similar amino acids are defined as I/L/V/M, D/E, S/T, A/G, N/Q, R/H/K and W/F/Y.

Plasmid constructions

The *rad1*⁺ cDNA was excised from pGR4-rad1⁺ (gift of S.E.Sorensen) with *Bam*HI and *Xba*I, the 3' recessed ends were filled in with Klenow polymerase, and the cDNA was blunt end ligated into the *Sma*I site of the *S.pombe* expression vector pART1 (39), to generate pART1-rad1⁺. The *HRAD1* open reading frame (ORF) was amplified with primers HRAD1-5 (GGACGGTCGACATGCCCCTTCTGACCCAA) and HRAD1-3 (ACGGATCCTCAAGACTCAGATTCAGG), and blunt end ligated into the *Sma*I site of pART1, to generate pART1–HRAD1. Orientation of the inserts within pART1 was determined by restriction enzyme digestion.

Schizosaccharomyces pombe culture and manipulations

Schizosaccharomyces pombe was cultured using standard techniques (40). The strains used in this study were Sp337, h^{+N} rad1::ura4⁺ leu1-32 ura4-D18; and Sp199, h^{+N} cdc25-22 rad1-1 leu1-32. Sp337 was generated by crossing 975 (40) with Sp267 (41), and Sp199 was generated by crossing SP32 (41) with SP1202 (41). Schizosaccharomyces pombe transformations were performed using the method of Okazaki *et al.* (42).

Radiation sensitivity and radiation-induced cell cycle delay

X-irradiation was delivered using a Clinac 2100 C/D with a 6 MV beam, at a dose rate of 0.24 Gy/s. UV radiation treatments were performed at 254 nm, with a dose rate of 1.8 J/m²/s. For viability assays, *S.pombe* was cultured to mid-logarithmic phase (5 × 10^{6} cells/ml) at 25°C, plated on minimal selective media at a density of 1000 cells per plate, and irradiated with the indicated dose of radiation. The plates were incubated at 30°C until colonies were easily visible. Relative viability was expressed as the number of treated versus untreated cells that were able to form colonies.

To assess radiation-induced checkpoint control, plasmids were transformed into a *cdc25-22 rad1-1* strain background. These cells were cultured to mid-logarithmic phase at 25 °C, plated on pre-warmed minimal selective plates, and incubated at 36 °C for 3 h to synchronize the cells in G₂. Immediately prior to release from 36 °C, the plated cells were irradiated with the indicated dose of UV radiation, transferred to liquid minimal selective media, and incubated at 25 °C. Samples were removed at the indicated times and fixed in 3.7% formaldehyde. Fixed cells were washed once with phosphate buffered saline (PBS), once with PBS containing 1% Triton X-100, and resuspended in PBS. The cells were then stained with 0.2 µg/ml 4'6-diamidino-2-phenylindole (DAPI) and viewed under a fluorescence microscope. Binucleate cells were scored as having passed mitosis.

Sensitivity to HU

Schizosaccharomyces pombe was cultured to mid-logarithmic phase at 32°C, and then HU was added to a final concentration of 12 mM. At the indicated times after the addition of HU aliquots of cells were removed, and plated on PM media at a density of 1000 cells per plate. The plates were incubated at 30°C until colonies had reached a suitable size for counting, and relative viability was assessed as described above for radiation sensitivity.

RESULTS

Isolation of the HRAD1 and MRAD1 genes

A search of the dBEST data base revealed an EST of interest obtained from a normalized and directionally cloned human cDNA library (43). The complementary strand of the EST appears to encode a predicted protein similar to the S.pombe rad1⁺ gene product. This ORF predicted a protein that is 30% identical and 57% similar over an 80 amino acid stretch, which represents approximately one quarter of the Rad1p protein. It is aligned closer to the C-terminal portion of the protein which is a moderately conserved region in the S.pombe rad1+, S.cerevisiae RAD17 and U.maydis REC1 gene products. The extent of homology in the region that the EST is aligned with S.pombe rad1⁺ is comparable to that of rad1⁺ and RAD17 (44). This same region contains nine identical residues between Rad1p, RAD17p and REC1p, of which seven are also present in the human EST. Based on the alignment and extent of sequence identity, this was evidence for the existence of a possible human homologue of S.pombe rad1⁺.

Because a positive orientation clone had not been identified in the original library, we chose to search other cDNA libraries for the *bona fide* human *rad1*⁺ homologue. A HaCaT (spontaneously transformed human keratinocyte) cDNA library in λ ZAP II was amplified by PCR using oligonucleotide primers directed against the putative *HRAD1* gene. The 399 bp PCR product generated using oligonucleotides A and B was subcloned into pBS KS⁻. Sequencing of the subclone revealed an insert of identical sequence to that of the original EST, confirming that the sequence of interest was present in the HaCaT cDNA library.

The screen of the HaCaT cDNA library yielded four positive clones. *In vivo* excision converted these λ cDNA vectors into pBS plasmids containing the cDNA insert. Sequencing indicated that all four contained the same cDNA. One of these, clone HRAD1-7, was slightly longer than the others and was chosen for further analysis.

The full-length HRAD1-7 clone was used to probe a mouse CB7 erythroleukemia cDNA library by low stringency hybridization. Five positives were identified, four of which were the same length, and one was slightly shorter than the others. Clone MRAD1-2.1 was chosen for further analysis.

Sequence analyses of the HRAD1 and MRAD1 genes

Full DNA sequences of both strands of the insert of clone HRAD1-7 showed that the cDNA was 1300 bp long with a 214 bp 5' untranslated region (UTR), an 846 bp coding region and a 240 bp 3' UTR (Fig. 1A). The 3' UTR contains a consensus AATAAA polyadenylation signal sequence. The ORF of *HRAD1* encodes a 282 amino acid polypeptide with 27% identity and 53% similarity to Rad1p. This is 41 amino acids shorter than the *S.pombe rad1*⁺ gene product.

Complete sequencing of both strands of clone MRAD1-2.1 identified a cDNA that was 1380 bp long with a 218 bp 5' UTR, an 840 bp coding region and a 322 bp 3' UTR (Fig. 1B). The 3' UTR contains a common variant of the consensus polyadenylation signal sequence (ATTAAA). However, no poly A tail is observed in this cDNA isolate. The ORF of *MRAD1* encodes a 280 amino acid polypeptide that is 90% identical and 96% similar to HRAD1p. An amino acid alignment (Fig. 2) shows that the sequence similarity of HRAD1p and MRAD1p to the other members of the Rad1p family extends over their entire lengths, suggesting that the isolated human and mouse cDNAs are full-length.

HRAD1 partially rescues the G₂ DNA damage checkpoint defects of *rad1* yeast mutants

The *HRAD1* ORF was subcloned into the *S.pombe* expression vector pART1 under control of the strong, constitutive *adh1*⁺ promoter. Expression of *HRAD1* in a *rad1::ura4*⁺ strain background increased the survival of these mutants following UV irradiation, to levels above that of the vector transformed control (Fig. 3A). However, this increase in viability did not reach the level of rescue that was obtained by expression of the wild type *rad1*⁺ gene (Fig. 3A). Expression of *HRAD1* also restored partial resistance to ionizing radiation (Fig. 3B).

In order to more rigorously examine if HRAD1 rescues the checkpoint defects of rad1 mutants, HRAD1 was expressed in a rad1-1 strain containing the temperature sensitive cdc25-22 allele. At the restrictive temperature of 36°C, these yeast arrest at the G₂/M transition point, due to their inability to activate the Cdc2 kinase. If cells blocked at the G₂/M transition are irradiated just prior to being released to the permissive temperature of 25°C, checkpoint proficient cells will undergo a dose dependent delay in entry into mitosis. Checkpoint deficient cells will enter mitosis without a noticeable delay. As shown in Figure 4A, the checkpoint

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GAATTCGGCA	CGAGCCGAGG	TGGAGGGCCG	GTCTGAAGAG	TGGCGGGGACT	GGCTTCACTT	60
CCTCCGCGGT	TCCTCGGAGC	CGCCTCGCTC	CTCTTCAGGG	ACTITIGCTGA	GAAGGGCTCT	120
CGGGCGTCCA	GACCCCACCG	CAAAGGTGTT	TGGCGATCCG	CCGAGAAGTT	GTTGGCCCCA	180
GGAGCATCCC	TCGGGGCCGA	ATGCGCAGTG	GACGATGCCC	CTTCTGACCC	AACAGATCCA	240
AGACGAGGAT	GATCAGTACA	GCCTTGTGGC	CAGCCTTGAC	AACGTTAGGA	ATCTCTCCAC	300
TATCTTGAAA	GCTATTCATT	TCCGAGAACA	TGCCACGTGT	TTCGCAACTA	AAAATGGTAT	360
CAAAGTAACA	GTGGAAAATG	CAAAGTGTGT	GCAAGCAAAT	GCTTTTATTC	AGGCTGGAAT	420
ATTTCAGGAG	TTTAAAGTTC	AGGAAGAGTC	TGTTACTTTT	CGAATTAATT	TAACTGTCCT	480
TTTAGACTGT	TTATCTATTT	TTGGATCAAG	TCCTATGCCA	GGGACTTTAA	CTGCACTTCG	540
AATGTGTTAC	CAAGGTTATG	GTTACCCTTT	GATGCTGTTC	CTGGAAGAAG	GAGGAGTGGT	600
GACAGTCTGC	AAAATCAATA	CACAGGAACC	TGAGGAGACC	CTGGACTTTG	ATTICTGCAG	660
CACCAATGTT	ATTAATAAAA	TTATTCTGCA	GTCAGAGGGG	CTCCGTGAAG	CATTTTCTGA	720
ATTGGATATG	ACGAGTGAAG	TCCTACAAAT	TACCATGTCT	CCTGACAAGC	CTTATTTCAG	780
GTTATCTACT	TTTGGAAATG	CAGGAAGTTC	CCACCTTGAC	TATCCCAAAG	ATTCTGATTT	840
GATGGAAGCA	TTTCATTGTA	ATCAGACCCA	AGTCAACAGA	TACAAGATTT	CCTTACTGAA	900
ACCCTCTACA	AAGGCATTAG	TCCTATCTTG	TAAGGTATCT	ATTCGGACAG	ATAACAGAGG	960
CTTCCTTTCA	TTACAGTATA	TGATTAGAAA	TGAAGATGGA	CAAATATGTT	TTGTGGAATA	1020
TTACTGCTGC	CCTGATGAAG	AAGTTCCTGA	ATCTGAGTCT	TGAGTATGAC	AATTCACTGA	1080
TATTTATGTG	TACATTTATG	ATAGATGAAG	TTCTTATTCT	GAGTACAGTA	CTCTTTGTCA	1140
TTTCATATTG	GATTTTCTAT	AGAGAAGAAG	CACAATGGGG	AAGATAGGAG	CAAGGTCATG	1200
TACCCTAATA	GTTACTATGT	TTTGTAAATC	CATTTTGTAG	AGGGCATGTA	AATAAA TGTT	1260
TTCCTGTAGT	CATAGATTAA	алалалалаа	AAAACTCGAG			1300
В.						
GAATTCGCGG	CCGCGCTTTT	TGGACGCTCA	GGTGTTCTT	GGGGCTGGGG	TGGCAGGGGC	60
GAATTCGCGG	CCGCGCTTTT	TGGACGCTCA	GGGTGTTCTT	GGGGCTGGGG	TGGCAGGGGC	60 120
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG	TGGACGCTCA CCACAGCCTG CGTTCTCCTG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT	GGGGCTGGGG CTCCCTTAGT TTTCACACAT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG	60 120 180
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCC ATG CCT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC	60 120 180 240
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA	60 120 180 240 300
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA	60 120 180 240 300 360
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA CAAGGTTACA	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT GTGGAGAATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGTG	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT GCAAGCAAAT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GCCTTTATTC	60 120 180 240 300 360 420
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGCTGACGT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA CAAGGTTACA GTTTCAGGAA	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT GTGGAGAATG TTTGTCATTC	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT AGGAAGAATC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT GCAAGCAAAT TGTTACTTTT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GCCTTTATTC CGAATTAACT	60 120 180 240 300 360 420 480
GAATTCGCGG TTTGAGGGGT CGCCAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGGCTGACGT TAACTATCCT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA CAAGGTTACA GTTTCAGGAA TTTTAGGACTGT	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT GTGGAGAATG TTTGTCATTC TTATCTATTT	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT AGGAAGAATC TTGGATCAAG	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT GCAAGCAAT TGTTACTTTT TCCTACACCA	TGGCAGGGGC GGCCGCTGTG CTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GCCTTTATTC GGAATTAACT GGGACTTGA	60 120 180 240 300 360 420 480 540
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGGCTGACGT TAACTATCCT CTGCGCTTCG	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA CAAGGTTACA GTTTCAGGAA TTTAGACTGT GATGTGTTAC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT GTGGAGAATG TTTGTCATTC TTATCTATTT CAAGGTTATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT AGGAAGAATC TTGGATCAAG GTCACCCACT	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT GCAAGCAAAT TGTTACTTTT TCCTACACCA GATGCTATTT	TGGCAGGGGC GGCCGCTGTG CTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GCCTTTATTC CGAATTAACT GGGACTTTGA CTAGAAGAAG	60 120 180 240 300 360 420 480 540 600
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGGCTGACGT TAACTATCCT CTGCGCTTCG GAGGAGTGGT	CCGCGCTTTT CCGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA CAAGGTTACA GTTTCAGGAA TTTAGACTGT GATGTGTTAC GACGGTCTGC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GCCATTCATT GTGGAGAATG TTTGTCATTC TTATCTATTT CAAGGTTATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT AGGAAGAATC TTGGATCAAG GTCACCCACT CTCAGGAGCC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCATGAC CGCCACGTGT GCAAGCAAAT TGTTACTTTT TCCTACACCA GATGCTATTT TGAGGAGACA	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GCCTTTACTC CGAATTAACT GGGACTTTGA CTAGAAGAAG	60 120 180 240 300 360 420 480 540 600 660
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGGCTGACGT TAACTATCCT CTGCGCTTCG GAGGAGTGGT ATTTCTGCAG	CCGCGCTTTT CCGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC TGTCTTGAAA GTTTCAGGAA TTTAGACTGT GATGGTTAC GACGGTCTGC CACCAATGTT	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GACAGTACT GTGGAGAATG TTTGTCATTC TTATCTATTT CAAGGTTATG AAAATTACCA ATGAATAAAA	GGGTGTTCTT GGCCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CCAAGTGTGT AGGAAGAATC TTGGATCAAG GTCACCCACT CTCAGGAGCC CTCAGGAGCC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCTTGAC CGCACACGTGT GGTAGCAATT TGTTACTTTT TCCTACACCA GATGCTATTT TGAGGAGCA GTCAGAGGGG	TGGCAGGGGC GGCCGCTGTG GTCCCGAAG CTCCTAACCC AACGTTAGA TTTGCTACCA GGCTTTATTC CGAATTAACT GGGACTTTGA CTAGAAGAAG CTGCGGGAAG	60 120 180 240 300 360 420 480 540 600 660 720
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAACGGAAT AGGCTGACGT TAACTAATCCT CTGCGCTTCG GAGGAGTGGT ATTTCTGCAG	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC CAAGGTTACA GTTCAGAA TTTAGACGT GATCGGTCTGC CACCAATGTT	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GAACAGTACT GTGGAGAATG TTTGTCATTC TTATCTATTT TTATCTATTT CAAGGTTATG ANAATAAA ATGAATAAAA	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCCTGGACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT TGGATCAAG GTCACCCACT TTGGATCAAG GTCACCCACT TTATCCTGCA	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC GGCAACGTGG GGAAGCAAAT TGTTACTTT TCCTACACCA GATGCTATT TGAGGAGACA GTCAGAGGGG GACGTGTCT	TGGCAGGGGC GGCGCTTGG CTCCGGAAG CTCCTAACCC AACGTTAGCA TTTGCTAACCA GGCTTTATTC CGAATTATC GGGACTTTGA CTAGAAGAG CTGGATTTGG CTCGCGGAAG	60 120 180 240 300 420 480 540 600 660 720 780
GAATTCGCGG TTTGAGGGGT CGCGCAGCGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAATGA AGGCTGACGTTCG CGCGCTTCG GAGGAGTGGT ATTTCTGCAG CCTATTTCAG	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTACA GATGTTCAGAA CAAGGTTAGA GTTTCAGGAA TTTAGACTGT GACGGTCGC CACCAATGTT GCTGGACATG GTTGCTCACT	TGGACGCTCA CCACAGCCTG GTTCTCCTG GGACAGTACT GCCATTCATTG GTGAGAGATG TTTGTCATTC TTATCTATTT CAAGGTTATG AAAATTACA ATGAATAAAA ACAGGTATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC TCAGAGAAACA CAAGAGAAACA TTGGATCAAG GTCACCACT TTGGATCAAG GTCACCACC TTATCCTGCA TCCTACGAACC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTTGAC CGCCACGTGT GCAAGCAAT TGCTACACCA GATGCTATTT TCCTACACCA GATGCTATTT TGAGGAGGACA GTCAGAGGGG CACTGTGTCT	TGGCAGGGGC GGCCGCTGTG CTCCGGAAG CTCCTAACCC AACGTTAAGA TTTGCTACCA GCGTTTAATC GGACTTTGA CTAGAAGAAG CTGGATTTTG CTCCGGGAAG CCTGACAAGC	60 120 180 240 300 420 480 540 600 660 720 780 840
GAATTCGCGG TTTGAGGGGT CGCCGACCGG AGGAGCATCC AGTACAATGA ATCCTCCCAC AAAACGGAAT TAACTATCCT CTCGCGCTTCG GAGGAGTGGT ATTCTGCAG CCTTTTCGAG CCTTTTCGA	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTACA GTTTCAGAA GTTTCAGGAA GTTTCAGGAA GTTGGTCGC CACCAATGTT GCTGGACATG GTTGGACATG	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GCCATTCATT GTGGAGAATG TTTGTCATTC CAAGGTTATG AAATTACCA ATGAATAAAA ACAGGTGATG TTTGGAAATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCTTGGACC CCTTAGTGGC TCAGAGAACA CAAGAGTGTGT AGGAAGATGT TTGGATCAAG GTCACGGAGCC TTATCCTGCA TCCTACAGAT CAGGAACTC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTTGAC CGCCACGTGT GCAAGCAATT TGTTACTTTT TGAGGAGCA GTCACAGGGG CACTGTGTCT CCATCTTGAC	TGGCAGGGGC GGCCGCTGTG CTCCCGAAG CTCCTAACCC AACGTTAGGA TTGCTACCA GCGTTTTATC CGAATTAACT GGGACTTTG CTGCGGAAGATTTG CTCCGGGAAG CTCCGGGAAG CTCCCAAGC TACCACCCAAGC	60 120 180 240 300 420 480 540 600 600 720 780 840 900
GAATTCGCGG TTTGAGGGGT CGCGGAGGGG AGGAGCATCC AGGAGCATCC AAAACGGAAT AGGCTGACGT TAACTATCCT GAGGAGTGGGT ATTCTGCAG CCTTTTCGGACT ATTCCGACTT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTAC CAAGGTTACA GATCGTTACA GATCGGTCTGC CACCAATGTT GTTGCACTG GTTGCTCACT GGTCGGACCCC	TGGACGCTCA CCACAGCCTG GCTCTCCCG GCACAGGT GCGACAGTACT GCGATCATTCATT TTGTCATTC TTATCTATTT CAAGGTTATG AAAATTACCA ATGAATAAAA ACAGTGATG TTTGGAAATG TTTGGAAATG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CAAAGTGTG AGGAAGAATC TTGGAAGAAATC TTGGACGAG CTCAGGAGCC TTATCTGCA ATAAGACCA ATAAGACCA	GGGCTGGGG CTCCCTTAGT TTTCACACAT ATCOATGCT CAGCCATGAC GGCACGAGT GGAAGCAAGT TGTTACTTT TCTTACACCA GTCAGAGGGG CACTGTGTCT CCATCTTGAC GGTCAACAGA	TGGCAGGGGC GGCCGCTGTG CTCCGAAGC AACGTTAGGA TTTGGTACCA GGCTTTATTC GGAATTAACT GGGATTTGG CTCGGAATTTG CTCGGGAAG CTGGAATTTG CTCCGGGAAG CTGGACAAGCT TATCCCAAAGCTGT	60 120 180 240 300 420 480 540 600 660 720 780 840 900 960
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACAATGA ATCCTCCAC AAAACGGAAT AGGCTGACGT TAACTATACCT CTGCGGCTCGG GAGGAGTGGG ATTCCTGCAG CCTATTCGCAG CCTATTCGAG ATTCCGACTT CGCTACGAAC	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCG AGAGGACTAC TGTCTTGAAA GTTTCAGGAA TTTAGACGT GATGGTGTTAC GACGGTCTGC CACCAATGTT GCTGGACATG GTTGTCTACT GGTGGAAGCC GCCCTCTACA	TGGACGCTCA CCACAGCCTG CGTTCTCCTG GACCACGGT GACCACGTACT GCCATTCATT GTGGAGAATG TTTGTCATTC CAAGGTTATG AAAATTACCA ATGAATAAAA ACAGGTATA TTTGGAAATG TTTGGAAATG TTTGCACTGTA AAGGCCTAG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CAAAGTGTT AGGAAGAATC TTGGATCAG GTCACCCACT CTCAGGACCCA TCTTACCGCA CTTACAGAA	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTTGAC GGCAGCAAAT TGTTACTTTT TGCTACACCA GATGCTATTT TGCTACACGA GTCAGAGGGG CACTOTGTCT CCATCTTGAC GGTCAACAGA TAAAGTGTCT TGAAGATGGG	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GGACTTTGG GGACTTTGA CTAGAAGAAC CTGGGGAAGATTTG CTCCGGGAAG CCTGACAAGC TACCCCAAAG TACAGCCGT ATCCCGGACAGC	60 120 180 240 300 420 480 540 600 660 720 780 840 900 960 1020
GAATTCGCGG TTTGAGGGT CGGCGAGGGGAGGA AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGAATT AGGCTGACGT TAACTATCCT CTGCGCTTCG AGGGATGGT ATTCTCGAG ATTCCGACTT GGCTACTGAA ATAACCGAGT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCAC TTCTTCTCCGG AGAGGACTAC GTTTCAGAA GTTTCAGAA GTTTCAGGAA TTTAGACTGT GACGTCTGC CACCAATGTT GTTGGAAGCC GTTGGTCACT GGTCGGAACCC GCCCTCTACA CTTCCTCTCC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG GACAGCGGT GACAGTACT GCATTCATT TTGTCATTC TTATCTATT CAAGGTTATG ANATTACCA ATGAATAAAA ACAGTGATG TTTGGAAATG TTTCACTGTA AAGGCACTAG TTACAGTACA	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CAAAGTGTGT AGGAAGAATC TTGGAACAAG GTCACCCACT TTATCCTGCA TACCTAGAACTC AGGAACTCC GTTATCCTG TGATTAGAAA AGGTCCTGA	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCCT CAGCCTGAC GCCACGCACGTGT TGTTACTTTT TGTAGCACAT GTCAGAGGGG CACTGTGTCT CCATCTTGAC GGTCATCAGA TGAAGATGGG GTCCTGAATA	TGGCAGGGGC GGCCGCTIGTG CTCCCGAAGC CTCCTAACCC AACGTTAGGA TTTGCTACCA GGACTTTGA CTGGAACTTTGA CTGGGACTTTGA CTCGGGAAG CCTGGCAAGC CCTGGCAAGC TACCAGGCGGT AACCGGACAG CAGATATGTT ATTCACTGAC	60 120 180 240 300 420 480 540 600 660 720 780 840 900 900 900 1020
GAATTCGCGG TTTGAGGGGT CGGCGAGCGG AGGAGCATCC AGTACATGA ATCTCTCCAC AAAACGGAT AGGCTGACGT ATTCTGCAG CCTATTCTGCAG CCTATTCTGCAG ATTCCGACTTC GCCACTGCAA ATTACCGACTT CGCCACCAGG TTGTGGAATA	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTACA GTTTCAGAA TTTAGACTGT GACGGTTGCC GACGGTCTGC CACCAATGTT GCTGGACATG GTTGTCTACT GGTGGAAGCC GCCCTCTACA GTTCTCTCTCC TTACTGCTGC TTCTCTCTCC	TGGACGCTCA CCACAGCCTG CGTLTCCTCG GACCACGTA GCCACCGT GCCATTCATT GTGGAGAATG TTTGTCATTC CAAGGTTATG AAAATTACCA ATGAATAAAA ACAGGTATAG TTTCACGTATA AAGGCACTAG TTTCACGTACA CCTGATGAAG	GGGTGTTCTT GCGCCAGGGG CAAGCAGGT ACCGTGGACC GCTTAGTGGC CCAGAGAACA CAAAGTGTT AGGAAGAACA CTCAGGAGCC TTGGATCAG GTCACGAACCCA CTTACCGCA CTTACCGCA CTTATCCTG AGGAAACTC ATAAGACCCA CTTATCCTG AGGATAGCACA CTTATCCTG	GGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTTGAC GGCAGCAAAT TGTTACTTT TGCTACACA GATGCTATTT TGAGGAGACA GTCAGAGGG CACTOFGTCT CCATCTTGAC GGTCACACA TAAAGTGCT TGAAGATGGG GTCTTGAAT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTAACCC GGAATTAATC CGGAATTAATC CGGAATTATG CTCGGGGAAG CCTGGACAGC CTCGCGGGAAG CCTGGACAGC CAGATATGTT ATCCCGGACAG CCTGGCTGTC	60 120 240 300 420 480 540 600 600 720 780 840 900 960 1020 1020
GAATTCGCGG TTTGAGGGT CGCGAGAGGG AGGAGCATCC AGTACAATGA ATTCTCCCAC AAAACGAATT AGGCTGACGATT GAGGAGTGGT ATTCTGCAG CCTATTCTGGAG CCTATTCTGGAG ATTCCGACTT CGCTACTGAA ATAACCGAGG TTGTGGGAATA TATTCTGTCC	CCGCGCTTTT CGGACGTACG GCCGAAGTCG TTCTTCTCGG AGACGACTAC GTTTCAGA GTTTCAGAC GTTTCAGAC GTTTCAGCA GTTGTAGCAC GCCGCTCAC GTGGAAGCC GCCCTCTACA CTTCCTCTCC TTACTGCTGC TTTTTTTTTT	TGGACGCTCA CCACAGCCTG GGTTCTCCTG GACCAGCGGT GACAGTACT GTGGAGAATG TTTGTCATTC TTATCTATTT CAAGGTTATG AAAATTACA ATGAAATAAA ACAGTGATG TTTCACTGTA AAGGCACTAG TTACAGTACA CCTGATGAGA TTCTCACGTACA	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGAACC GCTTAGTGGC TCAGAGAACA CAAAGTGTGT TTGGATCAAG GTCACCACT TTGGATCAAG GTCACCACT TTGGATCCTG ATAAGACCCA CTTAATCCTG TGATTAGAAA AAGTTCCTGA AAGTCCTGA	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTGAC GGCACGTGGC GGCACGTGG GGAGCAATTT TCCTACACCA GATGGTATTT TGAGGAGAGA GTCAGAGGGG GGTCATGACAA TGAGAGGGGT TGAAGAGTGGT TGAAGAGTGGC TGAAGAGTGGC GGTCTTGAACA	TGGCAGGGGC GGCCGCTGTG ACCTTAGCC AACGTTAGCA CTCCTAACCC AACGTTAGCA GGGACTTTGG CTGGAATTAACT GGGACTTTGG CTGGAATTTTG CTCGGGAAT CTGGACAAGC TACCAGACAG CAGATATGTT ATCCGCAAGC CCTGGCATGCC	60 120 240 300 420 480 600 660 720 780 900 960 1020 1080 11200
GAATTCGCGG TTTGAGGGT CGGGAGGGGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT AGGCTGACGT TAACTATCTC CGCGCTTCG GAGGAGTGGT ATTTCTGCAG CCTTATTCAG ATTCCGACTT GGCTACTGAA ATAACCGAGG TTATGGAATCA CCCGAGTGCT	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGACTAC TTTCAGAA CAAGGTTACA GTTTCAGAA CAAGGTTACA GTTGTGTTAC GCCGCAATGTT GCTGGACATG GCCCCTCACA GCCCCCTCACA CTTCCTCTCC TTACCGCGC CTCCCCCCC TACCGCGCC	ТGGACGCTCA ССАСАGССТG ССАСАGССТG ССАТСТССТG GACCAGTACT GTGGAGAATG TTTGTCATTC CAAGGTTATG TTATCTATT CAAGGTATA ACAGGTATA ACAGGTATA TTTGAAAATG TTTCACGTAAA CCTGATGACG CCAGCGCCCA CAGCCGCCA	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CCAGGAGACA CAAAGTGTGT TGGATCAAG GTCACGAGACC TTGGATCACGAG CTTATCCTGCA TCATACCAG CAGGAAACTC CAGGAACTCCTG AGACAGGGTT TCGATCACGG	GGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTTGAC GGCACGTGGC GCAGCAGAAAT TGTTACTTT TGTAGCAACA GTCAGAGGGG CACTGTACT TGAGGAGGG GTCAGAGGG GTCATGACT TGAAGATGGG TCTTGAACA AAATCACCT CGAATATTTC	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGCA GCCTTATTC CGAATTAACT GGGACTTGA CTGGATTTG CTGCGGGAAG CCTGGCATTGG CCTGGCATGC AACCCGAAA ATCCGGACAG CAGATATGTT ATTCACTGAC CCTGGCTTGTCCT	60 120 240 300 420 480 540 600 660 720 780 840 900 960 1020 1080 1140 1260
GAATTCGCGG TTTGAGGGT CGCGAGAGCATCG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGAAT TAACTATCCT CTGCGCTTCG GAGGAGTGGT ATTCTGCAG CCTATTCCAG CCTATTCCAG ATTCCGACTT CGCTACTGAA ATACCGACG TTGTGGAATA TATTCTGTCG CCGGAACTCA CCGGAGCGTT	CCGCGCTTTT CGGACGTACG GCCGANGTCG TTCTTCTCGG AGAGGACTAC GTTTCAGAA CAGGTTACA GATGTGTAG GACGTCTGC CACCAATGTT GCTGGACATG GTTGCACACG GCTCCTCTCC CCCCCCCCAC CTTCCTCTCTCTTACACCGCGCTTACAGCGCGCATTAAGAC GCGGATTAAGAC GCGGATTAAGAC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG AGCCGACGGT GACAGTACT TGGCGACGGT GCATTCATT TTGTCATTC TTATCTATTT CAAGGTTATG AAAATTACA ATGAAATAAA ACAGGTGATG TTTCACTGTA AAGGCACTAG TTTCACTGTA AAGGCACTAG TTACAGTACA CTGATAGACG	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CAAAGTGTT AGGAAGATGT TTGGATCAAG GTCACCACT CTCAGGAGCCT CTTACGGACCCA CTTATCCTGA AGACCCACTGA AGGTCCTGA AGGCACGGCT CGATGATTTC	GGGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTTGAC CGCACGTGAC CGCACGAAAT TGTTACTTT TGCTACACCA GATGCTATTT TGCTACACCA GTCATGGGAT TAAGGGGC CATCTTGAC GTCATGATGG TCATGATATTG GTCTTGAATATTG GAAATATTG	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGGA TTTGCTACCA GGACTTTGG CGGACTTTGA CTAGAAGAAG CTGGGGAAG CTGCGGGAAG CCTGCGGGACG TACCCCAAAG CTGCAGGACGT ATCCCGACAGC CCGGCGCGCC TGTCCTTTATA	60 120 240 300 420 540 600 660 720 840 900 900 900 900 900 1020 1140 1200
GAATTCGCGG TTTGAGGGT CGGGAGGGGAGGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGAAT TAACTATCCT CTGCGCTTCG GAGGAGTGGT ATTCTCGCAG CCTATTTCAGA CCTATTCAGA ATTACCGACT TTGTGGAATCA TGCGAATCA ATGCCAGGGTGCT GTGGCGATCCA	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCTCGG AGAGGACTAC GTTTCAGAA GTTTCAGAA GTTTCAGAA GTTTCAGAA GTTGCTACT GCCGCTCTACA GTCGGAACCC TTACTGCTACC TTACTGCTGCC TTACTGCTGCC TTACTGCTGCC CTCCTACA GCGCTCTACA GCCCTCTACA GCGCTCTACA GCGCTCTACA CTCCTCTACA CTCCTCTACA CTCCTCTACA CTCCTCTACA CCCCCTCTACA CCCCCTCTACA CCCCCTCTACA CCCCCTCTACA	TGGACGCTCA CCACAGCCTG GATCTCCTG GACCAGTACT GCCATCACT GCCATCATT TTGCATTC TTGCATATT CAAGGTTATT CAAGGTTATT AAAATTAACA ATGAATAAAA ACAGTGATG TTTCGAAATG TTTCGAAATG TTTCACGTACA CCTGATGAAG TTCTTTCACG CAGGCGGCCA CCTCTAACCT	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGTG ACCGTGGACC GCTTAGTGGC TCAGGGACA CAAGAGAGTGT AGGAAGATGTG TGGATCAAG GTCACCCACT TTGGATCAGA TCCTACAGAA TCCTACAGAA CCTTTATCCTGCA ATAAGACCCA CTTTATCCTGA AAGATCCTGA AGGACAGGGTT TCGAACTCAG GAATACCTGA CCACGACCGG TGATGATTC	GGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTGAC GGCACGTGGC GGCACGGGT GCAACAAAA TCCTACACA GTCAGAGGGG CACTGTGTC CCATCTGAC GGTCAACAGA TCAAAGGGG GTCTTGAATAG TCTCTGTAGC AAATTCACCT CGAATATTTG ATTTAGATT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG CTCCTAACCC AACGTTAGCA GCGTTAGCA GCGTTAATCC GGAATTAACT GGGACTTTG CTCGGGAAG CCTGACAAGCAG CCTGACAAGCAG TACCCGACAA ATCCGGACAG CCTGGCTTC CCTGCCTTCACT ATCCCTGCCT ATCCCTGCCT CCTGTCTTCATA GTATTAGAGAG	60 120 240 300 420 480 660 660 720 720 720 720 720 720 1020 1020 1020
GAATTCGCGG TTTGAGGGT CGGCGAGGGGGG AGGAGCATCC AGTACAATGA ATCTCTCCAC AAAACGGAAT TAACTATCCT CTGCGCTTCG GAGGAGTGGT ATTCTGCAG CCTTTTCGGA CCTATTCCAG CCTATTCCAG CCTATTCCAG CCTATTCCAG CTGGACATC CGGAACTCA CCGGAGTGCT ATGCGGAC	CCGCGCTTTT CGGACGTCAC GCCGAAGTCG TTCTTCTCGG AGAGGAGTACA GTTTCAGAA GTTTCAGGAA GTTTCAGGA GACGGTCTGC GACGGTCTGC GACGGTCAC GTTGTCTACT GGTGGACATCG GCCCTCTACT TTCTTTTTTTT CTTTGTAGAC GGGATTAAG GGGATTAAG GGGATTAAG GCGCTCTACC AAAATAGGAG TC	TGGACGCTCA CCACAGCCTG CGTTCTCCTG GACCACGTACT GCCATCACT GCCATCATT GTGGAGAATG TTTGTCATTC CAAGGTTATG AAAATTACCA ATGATAAAA ACAGGTAATA TTTGGAAAATG TTTCACTGTA AAGGCACTAG CCTGATGAAG CCGATGACG CAGGCTGGCC GCATGCGCCA	GGGTGTTCTT GCGCCAGGGG CAAGCCAGGT ACCGTGGACC GCTTAGTGGC CCAGAGAACA CAAAGTGTT AGGAAGAACA CTCAGGAGCC TTGGATCAG GTCACGAACTC ATAAGACCCA CTTATCCTG ATAAGACCCA CTTATCCTG AGGATAACTC AGGACGGGT TCGAACTCAG CCACGACCGG CCACGACCGG	GGGCTGGGG CTCCCTTAGT TTTCACACAT ATCCATGCT CAGCCTGAC GGCACGGAAAT TGTTACTTT TGAGGAGACA GTCAGAGGG CACTGTGCT CCTACACAG TCAGGGGC CATGTTGAC GGTCACAGA TAAAGTGTCT TGAAGATGGG TCTCGTAGC AAATTCACCT CGAATATTCG AAATTCACCT	TGGCAGGGGC GGCCGCTGTG CTTCCGGAAG TTTGCTAACCC AACGTTAGA CTGGATTAGC GGGACTTTGA CTGGATTTGA CTGGATTTGA CTGGAGAG CTGGATTGG CTGCGGGACA CCTGGCTGT ATCCCGACAGC CAGATATGTT ATCCGGACAG GCCTCTGCCT GCCTCTGCCT TGTCTTTATA	60 120 240 360 420 420 420 420 420 600 720 780 840 900 900 1020 1020 1020 1140 12200 1220 1320

Figure 1. Nucleotide sequences of *HRAD1* and *MRAD1*. Nucleotide sequences of the *HRAD1* (**A**) and *MRAD1* (**B**) cDNAs. The initiating and terminating codons are shown in bold. Consensus polyadenylation signals are underlined. Numbers to the right indicate the number of the last nucleotide on each line.

deficient vector transformed controls enter a synchronous mitosis within 100 min of being irradiated, regardless of the dose received. Checkpoint proficient yeast expressing Rad1p undergo the characteristic dose dependent delay in entry into mitosis (Fig. 4B). Yeast expressing HRAD1p also undergo a dose dependent delay in entry into mitosis (Fig. 4C). The dose dependence is not equal to that of cells expressing Rad1p, however, this is what one would expect for partial rescue.

Expression of *HRAD1* restores minimal resistance to HU in *rad1::ura4*⁺ yeast

Expression of *HRAD1* in Sp337 confers weak, but statistically significant resistance to the transient DNA synthesis inhibitor HU. However, this rescue is not nearly as high as that observed in other instances, such as *HRAD9* rescue of *rad9 S.pombe* mutants (27). As shown in Figure 5, *HRAD1* expressing cells lose viability with kinetics similar to that of the vector transformed control. Cells expressing wild type *rad1*⁺ remain viable for at least 6 h in HU (Fig. 5).

DISCUSSION

We have identified novel human and mouse genes that are structural homologues of the fission yeast $rad1^+$ checkpoint control gene. The sequence similarity extends over the entire



Figure 2. Amino acid sequence alignment of HRAD1p and MRAD1p with members of the Rad1p family. Amino acid alignment of HRAD1p, MRAD1p, *S.pombe* Rad1p (Sp_rad1p), *S.cerevisiae* RAD17p (Sc_RAD17p) and *U.maydis* REC1p (Um_REC1p). Numbers to the right indicate the numbering of the final amino acid on each line. Identical residues in \geq 80% of the sequences are highlighted in dark grey. Conserved residues (defined in Materials and Methods) in \geq 80% of the sequences are highlighted in light grey. Potentially important functional regions include the putative $3' \rightarrow 5'$ exonuclease domains (Exo I, II and III), and the leucine rich region (Leu rich), which have been previously defined for other members of the family (35,36).

coding regions, indicating that the isolated cDNAs are full length. Particularly high levels of conservation were seen in two of three putative exonuclease domains, as well as in the leucine rich region that have been previously defined (35). The extent of amino acid conservation between HRAD1p and Rad1p, 27% identity and 53% similarity, is comparable to that observed between Rad1p and RAD17p (23% identity, 50% similarity). Rad1p and RAD17p have been shown by independent means to be involved in checkpoint control in fission and budding yeast, respectively (44). In different regions, HRAD1p and MRAD1p appear more like each of Rad1p, RAD17p and REC1p. Together with the functional complementation of *rad1* mutants by *HRAD1*, and the extent and pattern of structural similarity within this family, *HRAD1* and *MRAD1* are highly likely to be involved in mammalian G₂ checkpoint regulation.

While it has been clearly demonstrated that REC1p is a $3' \rightarrow 5'$ exonuclease, it has also been demonstrated that this function is not required for checkpoint control by this protein (35). The sequence similarity between HRAD1p, MRAD1p and other members of the family over the exo II and exo III domains is high, but less so in the exo I domain. The role of the putative $3' \rightarrow 5'$ exonuclease in HRAD1p function is questionable at this point.

We were able to show that HRAD1 can partially rescue radiation sensitivity in *rad1* mutant yeast. This rescue is due to partial restoration of the G₂ checkpoint defect of these mutants, which is shown by the radiation-dose dependent delay experiment (Fig. 4). Checkpoint deficient vector transformed yeast begin to transit mitosis within 40 min of being released to the permissive temperature, regardless of the dose received. The checkpoint proficient yeast overexpressing Rad1p undergo a dose dependent delay in entry into mitosis. The unirradiated cells do not begin to transit mitosis until 60 min after release to the permissive temperature, which is 20 min later than the vector transformed cells. This difference is due to the additive effect of

two cell cycle delaying influences, the overexpression of Rad1p and the *cdc25-22* mutation, which is not completely wild type even at the permissive temperature. Neither overexpression of Rad1p nor the cdc25-22 allele alone is sufficient to cause the observed delay. Yeast rad1 mutants overexpressing HRAD1p also undergo a dose dependent delay in entry into mitosis. The observed delay is not equivalent to that of the Rad1p expressing cells, but this is what one would expect for partial rescue. The maximal percentage of cells passing mitosis in both Rad1p and HRAD1p expressing yeast is lower than yeast carrying empty vector. This is due to the quality of the synchrony of cells passing mitosis. As the delay increases, the synchrony of the cells begins to diminish. Therefore, the highest percentage of cells passing mitosis is observed in the checkpoint deficient cells, where release from the block is quick. Checkpoint proficient cells will gradually lose synchrony over time and the maximal percentage of cells passing mitosis is lower.

This partial complementation suggests that *HRAD1* is the human homologue of fission yeast *rad1*⁺. Cross species complementation by checkpoint genes has been demonstrated in other cases, but full complementation of all the defects of any particular mutant has not been observed. *HRAD9*, the human homologue of *S.pombe rad9*⁺, restores resistance to HU in *rad9* null mutants, but fails to rescue UV sensitivity (27). *FRP1/ATR* is the human homologue of *S.cerevisiae MEC1/ESR1* and *S.pombe rad3*⁺ (28,29,45). While *FRP1/ATR* will rescue some of the checkpoint defects of *MEC1/ESR1* mutants, it will not restore checkpoint proficiency to *rad3* mutants (29). Further analysis of *HRAD1* will be necessary to clearly define its role in human cell cycle checkpoint control.

In mammalian cells, the G_1 checkpoint is regulated in part by the *p53* and *ATM* genes, and defects in these genes have been associated with a variety of human cancers (3,4,8–11,16, 18,19,21,46,47). By contrast, very little is known about the



Figure 3. *HRAD1* expression restores resistance to DNA damage in *rad1::ura4*⁺ mutants of *S.pombe*. Sp337 was grown to mid-logarithmic phase in PM media, plated on PM plates, and irradiated with the indicated doses of radiation. Colonies were counted after 6 days and relative viability is expressed as the number of irradiated cells relative to unirradiated cells that were able to form colonies. (A) The UV dose versus survival curve for Sp337 carrying pART1 (\bullet), pART1-rad1⁺ (\blacksquare) or pART1-HRAD1 (\bullet). (B) The ionizing radiation dose versus survival curve. The symbols are the same as in (A). Both panels are the average of two independent experiments, each performed in duplicate. Error bars indicate standard error of the mean.

molecular control of the G_2 checkpoint in mammalian cells. Like yeast, mammalian cells will respond to DNA damage or incompletely replicated DNA by arresting the cell cycle in G_2 , prior to entry into mitosis. The presence of such a G_2 checkpoint has been shown to correlate with viability after exposure to radiation (48–52).

There are now three candidates for human G₂ checkpoint control genes: HRAD1, HRAD9 and FRP1/ATR, homologues of the $rad1^+$, $rad9^+$ and $rad3^+$ genes of S.pombe. To date, none of these has been shown to function in human G₂ checkpoint control, though HRAD1, HRAD9 and FRPI/ATR have been shown to rescue some of the defects in checkpoint deficient fission or budding yeast. Interestingly, BRCA1p co-localizes with the repair protein RAD51p, and both are found in regions of meiotic chromosomes similar to where FRP1p/ATRp is located (53,54). This spatial association with RAD51p and FRP1p/ATRp, and evidence that developmental arrest in Brca1 null mice is partially rescued by a p53 mutation indicates a role for BRCA1p in DNA damage repair (55). Genetic evidence from yeast indicates that rad1⁺, rad3⁺ and rad9⁺ are part of the same G₂ checkpoint control pathway, and may form a physical complex. This suggests that HRAD1p, as the homologue of Rad1p, may be part of a multisubunit complex that includes other checkpoint proteins including HRAD9p, FRP1p/ATRp, RAD51p and BRCA1p.



Figure 4. *HRAD1* expression restores dose dependent radiation-induced cell cycle delay to *rad1-1* mutants of *S.pombe*. Sp199 was grown to mid-logarithmic phase at 25 °C, synchronized at the G₂/M transition by a 3 h incubation at 36 °C, irradiated with the indicated doses of radiation (time zero), and released back to 25 °C. At the indicated time points after irradiation, cells were removed, fixed, stained with DAPI and viewed under the fluorescence microscope. The % cells passing mitosis for each sample is the number of binucleate cells expressed as a percentage of the total number observed. Greater than 100 cells were scored for each timepoint. (A–C) Sp199 carrying either pART1 (A), pART1-rad1⁺ (B) or pART1-HRAD1 (C). In each panel the doses were 0 J/m² (\blacklozenge), nd 30 J/m² (\bigstar). This figure is a representative example of three independent experiments.



Figure 5. *HRAD1* expression does not restore resistance to transient DNA synthesis inhibition in *rad1::ura4*⁺ mutants of *S.pombe*. Sp337 was cultured to mid-logarithmic phase at 32°C, HU was added to 12 mM (time zero), and aliquots of cells were removed at the indicated times and plated on PM media. Relative viability is expressed as the number of drug-treated versus untreated cells that were able to form colonies. The symbols represent Sp337 carrying either pART1 (\blacklozenge), pART1-rad1⁺ (\blacksquare) or pART1-HRAD1 (\bigstar). The experiment was performed in duplicate and the error bars represent standard error of the mean.

It has been shown that caffeine treatment partially restores sensitivity to radiation in cell lines which have lost G_1 checkpoint control through the loss of p53 (56,57). Presumably, the loss of the ability to undergo apoptosis in response to radiation in p53 mutant cells leads to radiation resistance. Caffeine is presumed to eliminate the G_2 checkpoint in these cells, leading to radiation-induced death by premature mitosis, typical of checkpoint defective cells. Directly targeting *HRAD1* or HRAD1p could be an efficient way of targeting human G_2 checkpoint control. If elimination of G_2 checkpoint function would restore sensitivity to radiation (i.e. $p53^{-/-}$ cells), there will be therapeutic benefits to inhibiting *HRAD1* or other G_2 checkpoint control genes and protein functions, in conjunction with radio- or chemotherapies.

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